Phenylalanine and Phenylglycine Analogues as Arginine Mimetics in Dengue Protease Inhibitors

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* Supporting Information

ABSTRACT: Dengue virus is an increasingly global pathogen. One of the promising targets for antiviral drug discovery against dengue and related flaviviruses such as West Nile virus is the viral serine protease NS2B-NS3. We here report the synthesis and in vitro characterization of potent peptidic inhibitors of dengue virus protease that incorporate phenylalanine and phenylglycine derivatives as arginine-mimicking groups with modulated basicity. The most promising compounds were (4-amidino)-L-phenylalanine-containing inhibitors, which reached nanomolar affinities against dengue virus protease. The type and position of the substituents on the phenylglycine and phenylalanine side chains has a significant effect on the inhibitory activity against dengue virus protease and selectivity against other proteases. In addition, the non-natural, basic amino acids described here may have relevance for the development of other peptidic and peptidomimetic drugs such as inhibitors of the blood clotting cascade.

INTRODUCTION

The dengue virus (DENV) belongs to the family of flaviviridae which also includes the West Nile virus (WNV), hepatitis C virus (HCV), and yellow fever virus (YFV). With 3.97 billion people living in areas with a high risk of DENV transmission and an estimated 390 million infections in 2010, DENV constitutes a global health problem. Since 2010, there were local transmissions reported in Croatia and France, indicating that DENV is spreading to new areas. Therefore, DENV is one of the most important mosquito-borne viruses. In severe cases, infection with DENV can cause hemorrhagic fever and dengue shock syndrome. Up to now, there is no specific drug treatment available. Four serotypes of the virus (DENV 1–4) have been extensively characterized, and a fifth serotype was described recently. The viral genome is an 11 kb positive-sense, single-stranded RNA which encodes a polyprotein with three structural (capsid, envelope protein, membrane protein precursor) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The nonstructural protein NS3 contains a protease and a helicase domain. The protease domain of NS3, also denoted as NS3pro, has a trypsin-like fold with a catalytic triad formed by Ser135, His51, and Asp75. Catalytic activity of NS3pro depends on the formation of a complex with the NS2B protein, which forms a part of the substrate recognition site. For biochemical studies, the NS2B-NS3pro complex is usually produced as a fusion protein in which the two constituents are connected by a glycine—serine linker.

Viral proteases are successful targets for the discovery and development of antiviral drugs. With protease inhibitors as key components in HIV therapy and novel drugs such as boceprevir, telaprevir, and simeprevir as HCV NS3 protease inhibitors, the clinical efficacy of viral protease inhibitors is established. Recently, nonpeptidic DENV protease inhibitors were described with good activity against DENV serotype 2 (DENV-2) in the low micromolar range. Behnam et al. described peptide hybrid inhibitors with activity in the nanomolar range. These peptidic inhibitors are derived from the protease recognition sequence. Peptidic inhibitors of DENV protease usually include the basic amino acids arginine and lysine as preferred residues in the P1, P2, and P3 positions. The guanidino group of arginine is a major determinant of specificity and activity. Replacement of this side chain with the intention to improve cellular permeability and metabolic stability of the inhibitors is usually detrimental to activity, and previous SAR studies demonstrated a loss of activity if arginine is replaced by nonbasic amino acids. Another approach to improve the inhibitory activity in vitro and in cellulo is the combination of a peptide sequence (RKn-NH2) with N-terminal “capping” groups,
leading to peptide hybrids.\(^{18,19}\) The tripeptide sequence Arg-Lys-Nle-NH\(_2\) capped with 2,4-thiazolidinedione derivatives showed increased activity and membrane permeability.\(^{19}\) Further exploration of the C-terminal residue identified phenylglycine as a non-natural amino acid that resulted in high inhibitory activity. By merging this optimized sequence to a 2,4-thiazolidinedione “cap”, the in vitro activity of the peptide hybrid inhibitors could be enhanced into the nanomolar range,\(^{15}\) and dual inhibitors of DENV and WNV protease can be obtained.\(^{20}\) However, these peptide hybrids were still not optimized for the S2 pocket with respect to target affinity. In addition, previously published docking simulations showed the importance of two basic residues, such as lysine in S1 and arginine in S2.\(^{19}\) Considering these studies, we screened for arginine-mimicking moieties to identify alternative fragments that favorably fit into the S2 pocket. We herein present the synthesis and structure–activity relationship (SAR) of DENV protease inhibitors using phenylalanines and phenylglycine analogues as substructure for arginine mimetics. Side chain modifications were performed on the peptides coupled to solid support, which increased yields and diminished formation of byproducts. These SAR explorations led to the discovery of novel and potent peptidic inhibitors of dengue protease that incorporate non-natural amino acids.

### RESULTS AND DISCUSSION

**Chemistry.** The chemical structure of the reported peptidic inhibitors here is based on the recently published inhibitor sequence R-Arg-Lys-Phg-NH\(_2\).\(^{15}\) All peptides were obtained by solid-phase peptide synthesis according to the Fmoc protocol. The unnatural amino acids 30b, 32a–b, and 34a–b were synthesized by protecting the α-amine position with a benzyol substituent. With the benzyol moiety as N-terminal cap, all synthesized non-natural amino acids could be coupled to the presynthesized Lys-Phg-sequence with good synthetic efficiency. Scheme 1 shows the synthetic approach for inhibitors 30b and 34b. Benzyol protection of (3-nitro)-L-phenylalanine followed by esterification\(^{21}\) gave the methyl ester 2, which was reduced to provide 3 as key intermediate for further syntheses. Compounds 4 and 6 were obtained by reaction of 3 with di-tert-butyl dicarbonate and bis-boc-pyrazole-1-carboxamidine,\(^{17}\) respectively. After methyl ester cleavage, the benzoyl-capped amino acids 5 and 7 were coupled to the presynthesized Lys-Phg-sequence according to the Fmoc protocol. An analogous synthetic strategy was applied for the synthesis of inhibitors 32a–b as shown in Scheme 2. Compounds 9a–b were obtained after benzyol protection and esterification. The following synthetic steps: palladium-catalyzed reaction at 40 °C, boc protection of the side chain, and hydrolysis of methyl ester afforded the amino acids 12a–b, which were successfully coupled to the indicated sequence. The syntheses of benzamidine-containing peptidic inhibitors as C-terminal arginine mimetics have already been described for furin\(^{22}\) and West Nile virus protease.\(^{23}\) For dengue protease inhibitors, there are currently no peptidic or peptidomimetic inhibitors that include amidinophenylalanine and amidinophenylglycine as arginine mimicking residues at the N-terminal position. Previous work by Cesar et al.\(^{24}\) described a method to synthesize amidines on Wang resin by solid phase support. According to this procedure, benzamidine-containing inhibitors...
Scheme 3. Synthesis of N-Bz-(4-cyano)-l-phenylglycine

(a) SOCl₂, MeOH; (b) BzCl, DIPEA, piperidine, DCM; (c) phenyl triflimide, DIPEA, DCM; (d) Ni(PPh₃)₂Br₂, PPh₃, KCN, Zn, acetonitrile, 65 °C; (e) Cs₂CO₃, MeOH/H₂O.

Scheme 4. Synthesis of Benzamidine-Substituted Peptides on Rink Amide Resin (Table 1 and Table 2)

(a) hydroxylamine, DIPEA, EtOH, 60 °C; (b) SnCl₂·2H₂O, DMF; (c) TFA, TIPS, H₂O.

Scheme 5. Synthesis of Compounds 39 and 40

(a) BzCl, DIPEA, DCM; (b) Pd/C, H₂, MeOH, HCl (aq); (c) 2-isovaleryldimedone, TFA, EtOH, 80 °C; (d) Cs₂CO₃, MeOH/H₂O; (e) piperidine, DMF; (f) Fmoc-Phe-OH, HATU, DIPEA, DMF; (g) Fmoc-Lys(boc)-OH, HATU, DIPEA, DMF; (h) HATU, DIPEA; (i) hydrazine, DMF; (j) ethylacetimidate hydrochloride, DIPEA, DMF; (k) TFA, TIPS, H₂O; (l) acetic anhydride, DMF.

Scheme 6. Synthesis of N-Benzoyl-(3-nitro)-l-phenylglycine (25) and N-Benzoyl-(3-guanidino)-l-phenylglycine (28)

(a) HNO₃, H₂SO₄; (b) SOCl₂, MeOH; (c) BzCl, DIPEA, DCM; (d) LiOH, H₂O/THF, 0 °C; (e) Pd/C, H₂, MeOH, HCl (aq); (f) bis-boc-pyrazole-1-carboxamidine, DMAP, DIPEA, MeOH.
were synthesized by loading Fmoc-protected cyano-l-phenylalanines and Lys-Phg substituted resin. The preparation of Lys-Phg was performed according to procedures for phenylalanine synthesis described previously (Scheme 3). Starting from (4-hydroxy)-l-phenylglycine, benzoyl and methyl ester protection furnished 14, followed by conversion to 15 using phenyl trityl chloride. Nickel catalyzed cyanation of 15 with potassium cyanide and final methyl ester hydrolysis afforded 17. To obtain benzamidine-containing inhibitors, the resin-bound peptides 31a–c were treated with hydroxylamine and disopropylethylamine to achieve the amidoxime as intermediate (Scheme 4).

The reduction of amidoximes with tin(II) chloride dihydrate afforded the benzamidine-containing compounds 33a–b and 35. Intermediate 19 was prepared by benzoyl protection of (4-nitro)-l-phenylalanine methyl ester followed by hydrogenation. 19 was further treated with isovalerylchloride and cesium carbonate to obtain 21. Inhibitors 39 and 40 were synthesized by coupling 21 to Lys-Phg on Rink amide resin. After removal of the protecting group 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl (div) by 2% hydrazine in DMF, the peptide-substituted resin was treated with ethylacetimidate to afford compound 39 (Scheme 5). 40 was prepared as shown in Scheme 5 by using acetic anhydride after div removal. Compound 28 was obtained as follows: 24 was prepared by nitrification of l-phenylglycine followed by benzoyl and methyl ester protections (Scheme 6). This intermediate was converted to 27 by reduction and subsequent reaction with bis-boc-pyrazole-1-carboxamidine. The cleavage of methyl ester afforded 28. Although different conditions and reagents were evaluated, coupling of 28 to the Lys-Phg sequence on solid support remained unsuccessful. However, coupling of 25 to this sequence using standard coupling conditions, followed by reduction on the solid support using tin(II) chloride and subsequent reaction with bis-boc-pyrazole-1-carboxamidine furnished the desired peptide 37 with a (3-guanidino)-l-phenylglycine moiety as arginine mimic (Scheme 7).

Derivatives 42a–b and 45a–b were synthesized as shown in Scheme 2 by using 3-trifluoromethyl benzoyl and 3-phenylpropionate as N-terminal caps.

Structure–Activity Relationships. A series of 16 tripeptides with the general sequence Bz-X-Lys-Phg-NH₂ (Figure 1), in which X refers to phenylalanine and phenylglycine analogues as arginine mimetics, was evaluated against DENV protease serotype 2. The SAR explorations were focused on the side chain of these residues and in particular on varying their basic functionalities. To confirm selectivity for DENV protease, all compounds were also evaluated against WNV protease, thrombin, and trypsin (see Table 1).

Nearly all compounds have a negligible activity against trypsin (see Tables 1 and 2). Compounds 34b and 37 possess a moderate inhibitory activity against trypsin, with IC50 values of 18.2 and 7.1 μM, respectively.

For most compounds, the activity against WNV protease was below 90% relative inhibition. The compounds 32a, 34b, and 40 have a notable activity against WNV protease with IC50 values ranging from 1.6 to 3.9 μM. We observed for 32a a 3-fold selectivity toward WNV protease compared to DENV protease. This underlines that WNV protease prefers substrates containing lysine at the P2 residue, which could be mimicked more convincingly by the aminomethylphenylalanine moiety. Moderate activity against WNV protease was also observed for compound 40.

In contrast, inhibition of dengue protease was generally much more pronounced by the compounds presented here (Table 1). However, the pyridyl substituent (29a) caused a decrease of inhibitory activity against DENV protease in comparison to arginine. A further extension of the phenylalanine substitution to para-substituted aniline (30a) and nitrile (31a) derivatives also led to practically inactive analogues. For the corresponding aminomethyl-substituted analogue (32a), an IC50 value (4.3 μM) in the range of the reference compound I (Table 1) was
observed. Although (4-aminomethyl)-L-phenylalanine is considered as one of the suitable replacements for basic side chains, there is no improvement of inhibitory potency compared to the previously published peptide hybrid by Behnam et al. Nevertheless, the aminomethyl moiety is less basic than the guanidino function of arginine. Previous approaches to replace arginine by lysine or less basic residues resulted in a loss of activity. The use of (4-guanidino)-L-phenylalanine as arginine replacement has already been described for a tetrapeptidic aldehyde dengue protease inhibitor, which, however, cannot be considered as a drug-like lead compound. With an IC₅₀ value of 580 nM, the (4-guanidino)-L-phenylalanine analogue had somewhat lower inhibitory activity than the benzamidine analogue (IC₅₀ = 440 nM, Kᵤ = 232 nM, Table 1). This 10-fold increase in inhibitory activity compared to reference suggests a preference for basic aromatic residues in S₂. Besides this, the insertion of amidinophenylalanine, as a more drug-like residue than 4-guanidinophenylalanine, offers the opportunity to develop amidoxime prodrugs. Amidoximes are less basic than the corresponding benzamidines and possess improved pharmacokinetic properties, in particular with respect to gastrointestinal bioavailability. Although benzamidines are often used as arginine mimicking groups, particularly in thrombin inhibitors and other protease inhibitors of the trypsin family, compound showed only minor effects on thrombin. The known serine protease inhibitor p-amino benzamidine was previously tested against the protease from

Table 1. Inhibitory Activity of N-Benzoyl-Capped Tripeptides with Several Phenylalanine Analogues as Arginine Mimetics; Effect of Modifications by Extending the Side Chain and Increasing Basicity (General Formula: Bz-X-Lys-Phg-NH₂)²

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<th>DENV</th>
<th>WNV</th>
<th>THR</th>
<th>TRY</th>
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<td></td>
<td>29a</td>
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</tr>
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</tr>
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<td></td>
<td>32a</td>
<td>90.5</td>
<td>4.32</td>
<td>0.83</td>
</tr>
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<td></td>
<td>33a</td>
<td>100.3</td>
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<td></td>
<td>34a</td>
<td>99.9</td>
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<td>0.11</td>
</tr>
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<td></td>
<td>39</td>
<td>98.8</td>
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<td></td>
<td>40</td>
<td>44.0</td>
<td>3.09</td>
<td>0.11</td>
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</table>

²Percent inhibition of the DENV NS2B-NS3pro protease serotype 2 (compd, 50 μM; substrate, 50 μM; K₅₀, 105 μM; enzyme, 100 nM). Further inhibitor concentrations were assayed for compounds percent inhibition ≥70%; IC₅₀ values against DENV NS2B-NS3pro protease serotype 2 (substrate concentration: 50 μM).³Percent inhibition of the West Nile virus (WNV) NS2B-NS3pro protease (compd, 50 μM; substrate, 50 μM; K₅₀, 212 μM; enzyme, 150 nM). ⁴Further inhibitor concentrations were assayed for compounds percent inhibition ≥90%; IC₅₀ values against WNV NS2B-NS3pro protease (substrate concentration, 50 μM). ⁵Percent inhibition of thrombin (compd, 25 μM; substrate, 50 μM; K₅₀, 16 μM; enzyme, 10 nM). ⁶Percent inhibition of trypsin (compd, 50 μM; substrate, 50 μM; enzyme, 1 nM). Ligand efficiency values are given in kcal/mol per non-hydrogen atom.
DENV serotype 2, without notable effect even at high concentration (20% inhibition at 100 μM).38 Ekonomiuk et al. identified two compounds with bisbenzamidine and guanidino-benzyl group by a fragment-based docking with IC₅₀ values of 2.8 and 34.2 μM against WNV NS2B-NS3pro, respectively.39 These small nonpeptidic compounds are potential lead candidates with ligand efficiencies of 0.34 and 0.28 for WNV protease. Compounds 33a and 34a showed only negligible activity against WNV, but we obtained ligand efficiencies of 0.21 and 0.2040 against DENV NS2B-NS3pro. Therefore, 33a and 34a have lower ligand efficiencies than the compounds reported by Ekonomiuk et al., however, regarding targets like DENV protease, larger compounds appear to be the only option to obtain significant binding energies due to the large and predominantly solvent-exposed active site.41

To confirm that para-amidinophenylalanine (33a) is the most promising N-terminal residue, we also investigated variations at the meta-position of the phenylalanine substituent. Shifting the side chain moieties from para- to meta-position resulted in loss of activity for the most active analogues (32b, 33b, and 34b). The effect of meta-substitution on pyridyl-(29b), amino- (30b), and nitrile residues (31b) in relation to the para-substituted derivatives is low. For compound 32b, a 3-fold lower target affinity compared to the para-substituted analogue 32a was observed. Surprisingly, the activity profile of the meta-substituted phenylalanine analogues 33b and 34b is significantly different. Whereas all para-substituted compounds have only negligible affinity against thrombin, 33b and 34b show an increase in thrombin activity, accompanied by a 7-fold and 3-fold loss of inhibitory potency against DENV protease compared to the para analogues. Previous work by Stürzebecher et al. already identified m-amidinophenylalanine derivatives as promising scaffolds for thrombin inhibitors,42 underlining these findings. In addition, we shortened the side chain of phenylalanine to phenylglycine, a (so far) underexplored non-natural amino acid. We synthesized 37, whereby (3-guanidino)-l-phenylglycine closely resembles the side chain length of arginine and includes the aromatic residue of phenylalanine analogues, which showed promising activities for DENV protease in this study. The exchange of the basic residue resulted in an inhibitory potency (IC₅₀ = 3.09 μM, Table 1) comparable to the reference compound I. Therefore, the (3-guanidino)-l-phenylglycine moiety can be considered as suitable replacement for the arginine side chain and may be relevant for the design of peptide-based inhibitors of other targets with arginine recognition motifs. Unexpectedly, the exchange of (4-amidino)-l-phenylalanine to (4-amidino)-l-phenylglycine resulted in the practically inactive compound 35 (relative inhibition: 24.8%, Table 1). A comparison of 35 and 37 underlines the preferred length of the side chain. To confirm that basic phenylalanine analogues are the most suitable alternative for arginine residues, we synthesized

### Table 2. Inhibitory Activity of Peptide Hybrids with Various N-Terminal Caps (General Formula R₁-R₂-Lys-Phg-NH₂) against DENV and WNV Proteases and Thrombin

<table>
<thead>
<tr>
<th>No</th>
<th>R₁</th>
<th>R₂</th>
<th>[%]²</th>
<th>IC₅₀ [μM]²</th>
<th>Hill Slope</th>
<th>[%]³</th>
<th>IC₅₀ [μM]³</th>
<th>[%]⁴</th>
<th>IC₅₀ [μM]⁴</th>
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<tr>
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<td>Arg</td>
<td>98.7</td>
<td>1.02 ± 0.05</td>
<td>0.95</td>
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<td>n.d.</td>
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<tr>
<td>42a</td>
<td>(4-amidino)Phe</td>
<td>99.8</td>
<td>0.21 ± 0.01</td>
<td>1.20</td>
<td>69.9</td>
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<tr>
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<td>0.91</td>
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<tr>
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<td>100.5</td>
<td>0.69 ± 0.02</td>
<td>1.09</td>
<td>42.2</td>
<td>n.i.</td>
<td>n.d.</td>
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<tr>
<td>44</td>
<td>Arg</td>
<td>95.9</td>
<td>2.70 ± 0.12</td>
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<tr>
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<td>100.8</td>
<td>0.26 ± 0.03</td>
<td>1.52</td>
<td>67.7</td>
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<td>4.11 ± 0.50</td>
<td>0.76</td>
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"Percent inhibition of the DENV NS2B-NS3pro protease serotype 2 (compd, 50 μM; substrate, 50 μM; Kᵥ, 105 μM; enzyme, 100 nM).37 IC₅₀ values against DENV NS2B-NS3pro protease serotype 2 (substrate concentration, 50 μM).3 Percent inhibition of the West Nile virus (WNV) NS2B-NS3pro protease (compd, 50 μM; substrate, 50 μM; Kᵥ, 212 μM; enzyme, 150 nM).% Percent inhibition of thrombin (compd, 25 μM; substrate, 50 μM; Kᵥ, 16 μM; enzyme, 10 nM).% Percent inhibition of trypsin (compd, 50 μM; substrate, 50 μM; enzyme, 1 nM). All measurements were carried out in triplicate. Standard deviations were ≤10%, n.d. = not determined.

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Compound 39 with a less basic functionality showed a 20-fold loss of activity, and the nonbasic (4-acetylamino)-I-phenylalanine analogue 40 was completely inactive against DENV protease.

The most promising tripeptide sequences H-(4-amidino)-Phe-Lys-Phg-NH₂ and H-(4-guanidino)Phe-Lys-Phg-NH₂ were merged with 3-trifluoromethyl benzoic acid and 3-phenyl propionic acid as alternative N-terminal caps. The combination of 3-trifluoromethyl benzoic acid and the sequence R-(4-amidino)Phe-Lys-Phg-NH₂ resulted in the most potent compound of this series (42a, IC₅₀ = 210 nM, Kᵢ = 139 nM against dengue protease, Table 2). A phenylpropionic acid cap caused a slight loss of activity (45a, IC₅₀ = 260 nM, Kᵢ = 160 nM, Table 2) but still gave a 1.5-fold more active inhibitor than 33a. Compared to the reference sequences 41 and 44, a 5- and 10-fold improvement by exchanging arginine to (4-amidino)-I-phenylalanine was achieved. Combination of the sequence R-(4-guanidino)Phe-Lys-Phg-NH₂ with the selected caps led to a moderate decrease in activity (43 and 46 vs 34a). These results lend further support to the hypothesis that 4-guanidinophenylalanine is less tolerated by dengue protease.

Almost all investigated compounds show negligible inhibition of thrombin, with the highly active dengue protease inhibitor 42a displaying an inhibition of 50.3% at 25 μM against thrombin. Replacement of the benzyl group by the above-mentioned, alternative caps in combination with the sequence R-(3-amidino)Phe-Lys-Phg-NH₂ led to a minor drop in activity against dengue protease (42b and 45b, Table 2). More conspicuous is the change of selectivity between dengue protease and thrombin. Whereas 42b is a very weak thrombin inhibitor, a significantly higher thrombin affinity was observed for the sequence R-(3-amidino)Phe-Lys-Phg-NH₂ with 3-phenyl propionate cap (45b, IC₅₀ value of 4.59 μM against thrombin, Table 2).

Docking Studies. For the docking studies, we used the structure of DENV protease serotype 3 (pdb: 3U1I) reported by Noble et al.⁴⁴ to obtain an idea of possible interactions of the inhibitors with the target protein. 3U1I is the only available structure of the NS2B-NS3 complex of dengue protease with a small-molecular cocystalized inhibitor. The serotype 2 and 3 proteases have an identical substrate specificity and a high degree of conservation of the active sites. We therefore considered it appropriate to use the 3U1I structure for modeling studies. Docking simulations were performed with a selection of compounds covering a wide range of activities. However, no significant differences in the binding mode or docking scores could be observed. We therefore conclude that docking/scoring simulations are unable to capture subtle differences in ligand recognition that are responsible for the variation of affinity, at least in the relatively high affinity range which we observe in the present data set. For visualization purposes, and as representative examples, we chose compounds 42a (highest affinity) and 43 (see Figure 2). As expected, the basic residues of both arginine mimetics are located in the S₁ pocket to interact with Asp75 by electrostatic interactions. This interaction is comparable to the placement of basic residues observed in previous docking studies¹⁵,¹⁹ and the inhibitor–enzyme complex in the 3U1I structure.⁴³ This finding also matches the SAR results that a basic residue is required in the S₁ pocket.

Aprotinin Assay. To obtain information about the binding mode and to exclude nonspecific binding, the competitive tryptophan fluorescence assay reported by Bodenreider et al. was used.⁴⁵ This assay discriminates between specific and nonspecific binders and relies on the intrinsic fluorescence of Trp50 near the active site of dengue protease. An inhibitor that binds specifically to the active site of the enzyme quenches the tryptophan fluorescence if the inhibitor absorbs radiation in the range of the tryptophan emission (305–360 nm). The fluorescence will be restored if aprotinin displaces the inhibitor from the active site. Aprotinin is a known competitive ligand of dengue protease with relatively high affinity and without any tryptophan residues.⁴⁵,⁴⁶ Therefore a restoration of Trp50 fluorescence by aprotinin indicates a competition between the inhibitors. However, none of the compounds listed in Table 1 and Table 2 showed sufficient absorption between 305

Figure 2. Docking results for compounds 42a (green) and 43 (magenta) with crystal structure of DENV NS2B-NS3 protease (pdb: 3U1I). (A) Solvent-accessible surface, colored according to the Kyte–Doolittle hydrophobicity of the underlying amino acid (hydrophobic, red; hydrophilic, blue). S₁, S₂, S₃, and S₃' stand for protease subsites occupied by ligand moieties. (B) Ribbon model of the protein in the same orientation as in (A) with highlighted side chains of the catalytic triad. The protease domain is colored in red and the cofactor in blue. The figures were generated using the Chimera software.⁵⁸

40. Compound 39 with a less basic functionality showed a 20-fold loss of activity, and the nonbasic (4-acetylamino)-I-phenylalanine analogue 40 was completely inactive against DENV protease.

The most promising tripeptide sequences H-(4-amidino)-Phe-Lys-Phg-NH₂ and H-(4-guanidino)Phe-Lys-Phg-NH₂ were merged with 3-trifluoromethyl benzoic acid and 3-phenyl propionic acid as alternative N-terminal caps. The combination of 3-trifluoromethyl benzoic acid and the sequence R-(4-amidino)Phe-Lys-Phg-NH₂ resulted in the most potent compound of this series (42a, IC₅₀ = 210 nM, Kᵢ = 139 nM against dengue protease, Table 2). A phenylpropionic acid cap caused a slight loss of activity (45a, IC₅₀ = 260 nM, Kᵢ = 160 nM, Table 2) but still gave a 1.5-fold more active inhibitor than 33a. Compared to the reference sequences 41 and 44, a 5- and 10-fold improvement by exchanging arginine to (4-amidino)-I-phenylalanine was achieved. Combination of the sequence R-(4-guanidino)Phe-Lys-Phg-NH₂ with the selected caps led to a moderate decrease in activity (43 and 46 vs 34a). These results lend further support to the hypothesis that 4-guanidinophenylalanine is less tolerated by dengue protease.

Almost all investigated compounds show negligible inhibition of thrombin, with the highly active dengue protease inhibitor 42a displaying an inhibition of 50.3% at 25 μM against thrombin. Replacement of the benzyl group by the above-mentioned, alternative caps in combination with the sequence R-(3-amidino)Phe-Lys-Phg-NH₂ led to a minor drop in activity against dengue protease (42b and 45b, Table 2). More conspicuous is the change of selectivity between dengue protease and thrombin. Whereas 42b is a very weak thrombin inhibitor, a significantly higher thrombin affinity was observed for the sequence R-(3-amidino)Phe-Lys-Phg-NH₂ with 3-phenyl propionate cap (45b, IC₅₀ value of 4.59 μM against thrombin, Table 2).

Docking Studies. For the docking studies, we used the structure of DENV protease serotype 3 (pdb: 3U1I) reported by Noble et al.⁴⁴ to obtain an idea of possible interactions of the inhibitors with the target protein. 3U1I is the only available structure of the NS2B-NS3 complex of dengue protease with a small-molecular cocystalized inhibitor. The serotype 2 and 3 proteases have an identical substrate specificity and a high degree of conservation of the active sites. We therefore considered it appropriate to use the 3U1I structure for modeling studies. Docking simulations were performed with a selection of compounds covering a wide range of activities. However, no significant differences in the binding mode or docking scores could be observed. We therefore conclude that docking/scoring simulations are unable to capture subtle differences in ligand recognition that are responsible for the variation of affinity, at least in the relatively high affinity range which we observe in the present data set. For visualization purposes, and as representative examples, we chose compounds 42a (highest affinity) and 43 (see Figure 2). As expected, the basic residues of both arginine mimetics are located in the S₁ pocket to interact with Asp75 by electrostatic interactions. This interaction is comparable to the placement of basic residues observed in previous docking studies¹⁵,¹⁹ and the inhibitor–enzyme complex in the 3U1I structure.⁴³ This finding also matches the SAR results that a basic residue is required in the S₁ pocket. Moreover, the docking study shows the necessity of the phenylalanine residue. Whereas a p-amidinophenylglycine residue (Table 1, compound 35) shows almost no inhibitory activity, residues like p-amidinophenylalanines completely occupy the S₁ pocket, suggesting that the (4-amidino)-I-phenylglycine residue could be sterically hindered. This can explain the remarkable preference for the substituted phenylalanine residues. The side chain of lysine is placed in S₁ and the N-terminal cap is in the region of S₃. The phenylglycine residue is placed near the S₃' pocket and can interact with a hydrophobic surface.

Aprotinin Assay. To obtain information about the binding mode and to exclude nonspecific binding, the competitive tryptophan fluorescence assay reported by Bodenreider et al. was used.⁴⁵ This assay discriminates between specific and nonspecific binders and relies on the intrinsic fluorescence of Trp50 near the active site of dengue protease. An inhibitor that binds specifically to the active site of the enzyme quenches the tryptophan fluorescence if the inhibitor absorbs radiation in the range of the tryptophan emission (305–360 nm). The fluorescence will be restored if aprotinin displaces the inhibitor from the active site. Aprotinin is a known competitive ligand of dengue protease with relatively high affinity and without any tryptophan residues.⁴⁵,⁴⁶ Therefore a restoration of Trp50 fluorescence by aprotinin indicates a competition between the inhibitors. However, none of the compounds listed in Table 1 and Table 2 showed sufficient absorption between 305
and 360 nm, and these were therefore not suitable for the aprotinin competition assay. We therefore synthesized inhibitor 47 (Table S1), with the most active sequence of (4-amidino)Phe-Lys-Phg-NH₂ and 4-aminobenzoic acid as N-terminal cap. Abz is a commonly used building block of substrates for fluorescence-based assays. With an IC₅₀ value of 1.8 μM, the activity is weaker than the most active compounds 42a and 45a, but nevertheless 47 can be considered as a representative example. 47 causes a concentration-dependent nonlinear decrease of the Trp fluorescence in dengue protease (Figure S1). Addition of aprotinin causes a restoration of fluorescence. This indicates a specific binding of the inhibitors at the active site. Furthermore, the Hill slopes between 0.8 and 1.7 (Table 1 and 2) indicate a specific inhibition.

**Antiviral Activity in Cell Culture and Membrane Permeability.** The antiviral activity of five selected compounds, shown in Table 3, was determined in dengue virus serotype 2 infected human hepatocarcinoma cells (Huh-7) at a compound concentration of 50 μM (plaque assay). No cytotoxic effects were observed at this concentration of test compounds. The highest antiviral activity was observed for 45a with a reduction of virus replication of 58.8%, whereas the reference I and 33a, 42a, and 46 showed no relevant antiviral activity (percent inhibition <50%). This may be due to the basic residues of (4-amidino)-L-phenylalanine and (4-guanidino)-L-phenylalanine which increase the polarity of the compounds and may hinder cellular uptake.

Fifteen compounds, including the five compounds tested in cells, were screened for their permeability with the parallel artificial membrane permeability assay (PAMPA) procedure. PAMPA is an easily applicable, in vitro permeability model that predicts the passive biomembrane permeability of substances. None of the tested compounds had a detectable, passive membrane permeability (Table S2). The weak permeability can be explained by the polarity of peptidic compounds with two positively charged residues in P₁ and P₂. Lack of passive permeability may partially explain the low antiviral activity in these cells. This suggests that further consideration should be given to structural changes which increase the metabolic stability of the compounds such as modifications within the peptide backbone structure.

**Metabolic Stability of Selected Compounds in Liver Microsomes.** Peptidic compounds are sensitive toward metabolic clearance by enzymes in blood, kidney, liver, and other organs. Hepatic metabolism is a major elimination route and can be assayed with liver microsomes. These contain phase I metabolic enzymes which generate polar metabolites by hydrolysis, reduction, and oxidation. We studied the metabolic stability of compounds 45a and 46 in vitro using liver microsomes from rats, along with testosterone as reference compound. The samples were incubated for 30 min at 37 °C, and the loss of the parent compound was monitored. The half-times were: 45a, 22 min; 46, 18 min; testosterone, 63 min. Given the short halftime of the compounds in liver microsomes, the lack of activity in the hepatocyte-based viral replication assay may be due to fast metabolic clearance in these cells. This suggests that further consideration should be given to structural changes which increase the metabolic stability of the compounds such as modifications within the peptide backbone structure.

**CONCLUSION**

In this study, we evaluated the suitability of para- and meta-substituted phenylalanine and phenylglycine analogues as non-natural fragments for the discovery of dengue protease inhibitors. Straightforward syntheses on Rink amide resin furnished novel, peptidic dengue protease inhibitors with arginine-mimicking side chains. Using reactions on solid support, we obtained all synthesized peptides in high purity through a fast and elegant method. We found that the exchange of arginine to p-amidinophenylalanine results in a 10-fold more potent peptidic inhibitor (33a) for dengue protease compared to reference I. Incorporation of the (4-amidino)-L-phenylalanine residue leads to a more potent inhibitor with higher ligand efficiency that does not contain the potentially promiscuous 2,4-thiazolidinedione “cap”. The most potent dengue protease inhibitors 42a and 45a showed lower activity against WNV protease and no significant activity against thrombin and trypsin. An unexpected but significant observation is the reduced activity of meta-substituted phenylalanine analogues. Despite this, we identified (4-acetylamino)-L-phenylalanine as potential, nonbasic arginine replacement for West Nile virus protease inhibitors. In summary, we present optimized peptidic inhibitors of dengue protease with low promiscuity. Future work will aim on the further increase of metabolic stability.

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**Table 3. Results of Dengue Virus Plaque Assay for Selected Compounds (General Formula R₁-R₂-Lys-Phg-NH₂)**

<table>
<thead>
<tr>
<th>No</th>
<th>R₁</th>
<th>R₂</th>
<th>IC₅₀ [µM]⁺</th>
<th>Titer Reduction [%]⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Arg</td>
<td></td>
<td>3.3¹³</td>
<td>40.3 ± 2.7</td>
</tr>
<tr>
<td>33a</td>
<td>(4-amidino)Phe</td>
<td></td>
<td>0.44 ± 0.01</td>
<td>32.4 ± 4.2</td>
</tr>
<tr>
<td>42a</td>
<td>(4-amidino)Phe</td>
<td></td>
<td>0.21 ± 0.32</td>
<td>38.2 ± 1.3</td>
</tr>
<tr>
<td>45a</td>
<td>(4-amidino)Phe</td>
<td></td>
<td>0.26 ± 0.03</td>
<td>58.8 ± 0.0</td>
</tr>
<tr>
<td>46</td>
<td>(4-guanidino)Phe</td>
<td></td>
<td>0.89 ± 0.02</td>
<td>8.8 ± 4.2</td>
</tr>
</tbody>
</table>

⁺IC₅₀ values against DENV NS2B-NS3pro protease serotype 2 (substrate concentration, 50 µM). Percentage inhibition of viral replication in Huh-7 cells (compd, 50 µM).
target affinity and selectivity, along with the development of a prodrug strategy for arginine mimetics to improve pharmacokinetic properties and antiviral activity in cells.

**EXPERIMENTAL SECTION**

DENV and WNV Protease Expression and Purification. The dengue virus (serotype 2) and West Nile virus NS2B-NS3pro proteases were expressed and purified according to the protocol described before.50

**Protease Assays.** All protease assays were performed in 96 well V-bottom plates (Greiner Bio-One, Germany) and monitored using a BMG Labtech Fluostar OPTIMA microtiter fluorescence plate reader. The activity of the enzymes were determined as the slope per second (RFU/s) and monitored for 15 min. All determinations of percentage inhibition were calculated in relation to a positive control (without inhibitor) and performed in triplicate.

DENV and WNV protease assays were performed as reported before.18,19,51,52 In short, the continuous enzymatic assay was performed at an excitation wavelength of 320 nm and monitored at an emission wavelength of 405 nm. The assay buffer was used as described before as 50 mM Tris-HCl (pH 9.0), 10% ethylene glycol, and 0.0016% Brij 58.50 The inhibitors (final concentration: 50 μM) were preincubated for 15 min with DENV protease (100 nM) or WNV protease (150 nM), followed by the addition of substrate to initiate the reaction (final concentration: 50 μM). FRET substrates Abz-Nle-Lys-Arg-Ag-Ser-3([NO₂]₃)Tyr and Abz-Gly-Leu-Lys-Arg-Gly-Gly-3([NO₂]₃)-Tyr were used for DENV and WNV protease, respectively.

The thrombin assay was performed as described previously.19,54 In brief, continuous fluorimetric assay were performed at an excitation wavelength of 355 nm and emission wavelength of 460 nm. The assay buffer was used according to the literature as 50 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.05% Tween 20.55 The inhibitors (final concentration: 25 μM) were preincubated with thrombin (10 nM) for 15 min. The addition of boc-Val-Pro-Arg-AMC substrate (final concentration: 50 μM; Bachem, Germany) initiated the reaction.

The inhibition of catalytic activity of trypsin was determined by a continuous fluorimetric assay at an excitation wavelength of 355 nm and emission wavelength of 460 nm. The inhibitors (final concentration: 50 μM) were preincubated with trypsin (1 nM) for 15 min by using the buffer described for the thrombin assay. The addition of boc-Val-Pro-Arg-AMC substrate (final concentration: 50 μM; Bachem, Germany) initiated the reaction.

**IC₅₀ and Kᵢ Determination.** The assays were generally performed as described before. Further inhibitor concentrations (IC₅₀) were performed for compounds with a percent inhibition of at least 70%. The determination of IC₅₀ was performed by using different inhibitor concentrations (method A: 0, 0.125, 0.25, 0.5, 1, 2, 5 μM; method B: 0, 0.5, 1, 2, 3, 4, 5, 6 μM; method C: 0, 2.5, 5, 10, 15, 20, 25, 50 μM) and 50 μM substrate in triplicate. IC₅₀ values were calculated by Prism 6.0 (Graphpad Software, Inc.). Kᵢ determination was performed by using inhibitor concentrations of method A and substrate concentrations of 50, 100, 150, and 200 μM in triplicate. Kᵢ values were determined by plotting IC₅₀ values against substrate concentrations (Cheng–Prusoff equation).53,55,56

**Docking.** The calculations were performed on an Intel(R) Core(TM)2 Quad CPU Q9450 @ 2.66 GHz running open SuSE 11.0, using GOLD 5.2 and its graphical interface Hermes 1.6.27 The ligands were prepared using Chem3D Pro (PerkinElmer). The crystal structure of the NS2B-NS3 protease of DENV serotype 3 in complex with a tetrapeptidyl aldehyde was extracted from the dimer structure 3U1E.33 All waters and sulfate ions were removed, and the structure was further prepared using DoCK Prep (Chimera).34 All hydrogens were added, and the tetrapeptidyl aldehyde ligand was extracted from the structure. The binding site was defined in a radius of 10 Å around the extracted ligand. Ten different solutions were calculated for every ligand, and the docking results with the highest (42a) and second-highest (43) GOLD scores were aligned and visualized using Chimera.58

**Aprotinin Competition Assay.** The trypsinophan quenching assay was performed as described by Bodenreider et al.43 DENV protease (4 μM) was incubated with inhibitor 47 at different concentrations (5, 10, 20, 30, and 50 μM) by using the assay buffer as described previously. Moreover, the protease was incubated with the inhibitor (50 μM) and aprotinin (10 μM) together. Furthermore, as a negative control, the auto-fluorescence of the enzyme and aprotinin without inhibitor (0 μM) at the same concentrations was determined. The fluorescence of the assay samples was monitored on a Tecan Safire II instrument using an excitation wavelength of 280 nm and an emission wavelength of 320 nm. All determinations were performed in triplicate.

**Cell Viability Assay (Cytotoxicity Assay).** Huh-7 cells were seeded into 96-well plates (Greiner BioOne, Germany) in 50 μL of DMEM (Dulbecco’s Modified Minimal Essential Medium (Life Technologies, Germany)) (104 cells/well) supplemented with 10% fetal bovine serum. After overnight incubation at 37 °C, the medium was replaced with DMEM containing the respective concentration of the tested compound. Each concentration was assayed in triplicate. Cells were infected immediately with WT DENV serotype 2 with an MOI (multiplicity of infection) of 1 in the presence of the compound. After incubation for 48 h at 37 °C, the cells were harvested and the triplicates were pooled and stored at −80 °C. Then 50 μL of fresh DMEM was added to the cells and cell viability was determined using Cell-Titer Glo Luminescent Viability Assay. The highest nontoxic concentration was used for determination of the virus yield reduction by plaque assay using Vero E6 cells.

**Virus Titer Reduction Assay (Plaque Assay).** Vero E6 cells were seeded into 24-well plates (Greiner BioOne, Germany) with a density of 2.5×10⁴ cells per well in DMEM supplemented with 10% fetal bovine serum. After overnight incubation at 37 °C, the cells were inoculated with the virus-containing supernatants that were diluted with DMEM ranging from 10⁻¹ to 10⁻⁶ prior to infection. After incubation of the cells with 100 μL of the virus-containing dilution at 37 °C with agitation for 1 h, the medium was removed and 1 mL of plaque medium was added. After further incubation for 7 days at 37 °C, the cells were fixed with 5% (v/v) formaldehyde for 2 h, stained with 1% (w/v) crystal violet in 10% (v/v) ethanol, and plaques were counted. The virus titer reduction was calculated as percentage of plaques relative to plaques of untreated control.

**Parallel Artificial Membrane Permeability Assay (PAMPA).** PAMPA was performed as described before.19 In short, precoated PAMPA plate systems (BD Bioscience, Germany) were used to analyze permeability coefficient of compounds 32a–b, 33a–b, 34a–b, 37, 39, 40, 42a–b, 43, 44, 45a–b, and 46. The measurements were performed on Jasco HPLC-system with UV detector and RP-18 column (Reprosil Pur-ODS, Dr. Maisch GmbH, Germany; 3 μm, 50 mm × 2 mm) using the method: eluent A, water (+0.1% TFA); eluent B, acetonitrile (+0.1% TFA); flow rate, 1 mL/min; gradient, 1% B (0.2 min), 100% B (3.5 min), 100% B (4.5 min), 1% B (4.6 min), 1% B (5 min). Phosphate buffered saline (PBS) was used for all experiments. Calibration curves (correlation coefficients were found to be at least 0.9) were generated for all compounds and references (amiloride, caffeine, famotidine, furosemide, and phenytoin)46) at 10, 25, 50, 100, and 200 μM. PAMPA donor plate were filled with 300 μL of the 200 μM compound solutions in PBS in triplicate and 200 μL of PBS were added to wells of PAMPA acceptor plate. The plate system was incubated at room temperature for 5 h. The analysis was performed with HPLC by transferring the solutions of donor and acceptor plates into in 96-well polystyrene U-bottom plates (Greiner Bio-One, Germany) before. The concentrations were calculated using the generated calibration curves. Permeability (Pₐ) and the mass retention (R) were determined as described in literature.15

**Metabolic Stability in Liver Microsomes.** Pooled liver microsomes from male Sprague–Dawley were purchased from Sigma-Aldrich (Germany). To determine the metabolic stability, liver microsomal proteins (0.2 mg/mL) were supplemented with NADPH (5 mM) in Dulbecco’s Phosphate Buffered Saline (DPBS (Life Technologies, Germany)) and preincubated at 37 °C for 15 min. Additionally, test compounds (100 μM) were added and incubated at 37 °C for 30 min. Aliquots were removed at various time points (0, 2,
5, 10, 20, 30 min). The reaction was terminated by the addition of acetonitrile, and the samples were cooled with ice for 15 min before centrifugation (4500g at 4 °C for 15 min). The supernatants were used for further analysis. The loss of parent compound was monitored by HPLC on a Jasco HPLC system with UV detector and RP-18 column (ReproSil-Pur-ODS, Dr. Maisch GmbH, Germany, 3 μm, 50 mm × 2 mm) using the method: eluent A, water (+0.1% TFA); eluent B, acetonitrile (+0.1% TFA); flow rate, 1 mL/min; gradient, 1% B (0.2 min), 100% B (3.5 min), 100% B (4.5 min), 1% B (4.6 min), 1% B (5 min). Calculation of the metabolic stability was determined by dividing the peak areas of the unaltered parent compound in the metabolism sample by the peak areas of the parent compound in the reference sample. The activity of the microsomal preparations was verified by using a positive control (testosterone).

**Chemistry.** All chemicals were purchased from Sigma-Aldrich (Germany) and Alfa Aesar (Germany) with analytical grade. Amino acids were purchased from Alfa Aesar (Germany), Carbolution Chemicals (Germany), Orpegen (Germany), Iris Biotech (Germany), and Sigma-Aldrich (Germany). Reaction controls were performed by thin layer chromatography on Merck silica gel plates 60 F254 with UV detection and ninhydrin reagent. Flash chromatography was performed on a Biotage IsolaOne purification system. NMR spectra were recorded on Varian NMR instruments at 300 or 500 MHz in CDCl3, CD3OD, acetone-δ6. Chemical shifts are given in parts per million (ppm), and nondeuterated solvents were used as internal standard. Coupling constants (J) are given in hertz (Hz). HR-ESI mass spectrometry was performed on a Bruker microOTOF-Q II instrument. The purity was determined by analytical HPLC on a Biotage HPLC system with UV detector (method A) and an Agilent 1200 HPLC system with MWD detector combined with a Bruker microOTOF-Q II instrument (method B). A ReproSil-Pur-ODS-3 column, Dr. Maisch GmbH, Germany, 5 μm, 50 mm × 2 mm was used for HPLC. The conditions for method A were: eluent A, water (+0.1% TFA); eluent B, acetonitrile (+0.1% TFA); flow rate, 1 mL/min; gradient, 1% B (0.2 min), 100% B (3.5 min), 100% B (4.5 min), 1% B (4.6 min), 1% B (5 min). The conditions for method B were: eluent A, water (+0.1% formic acid); eluent B, acetonitrile (+0.1% formic acid); flow rate 0.3 mL/min, gradient, 5% B (1 min), 95% B (6 min), 95% B (10 min), 5% B (10.1 min), 5% B (12 min). UV detection was performed at 254 nm for both methods.

**General Procedures. Procedure A: Synthesis of Peptide Hybrids.** N-Terminal peptide hybrids were synthesized as described previously with some modifications. In short, the peptide sequence was synthesized analogously to the Fmoc protocol using Rink amide resin and 3.0 equiv of Fmoc protected amino acids, 5.0 equiv disopropylethylamine, and 3.0 equiv HATU as coupling reagent. After Fmoc removal and coupling steps, the resin was washed with DMF, DCM, and again DMF, respectively. The benzoyl protected amino acids (2.0 equiv) were coupled with HATU (2.0 equiv) and disopropylethylamine (2.2 equiv) to the dipeptide sequence. The peptides were cleaved from the resin by standard procedure (92.5% TFA, 5.0% TIPS, and 2.5% water), precipitated in cold diethyl ether, and purified by preparative RP-HPLC on an AKTApurifier, GE Healthcare (Germany) with an RP-18 column (Rephosphor, Dr. Maisch GmbH, Germany, C18-DE, 5 μm, 30 mm × 16 mm and 120 mm × 16 mm). All peptides were freeze-dried and characterized by HR-ESI and 1H NMR for selected compounds (analytical data are provided in the Supporting Information). All evaluated peptide hybrids were obtained with a purity of at least 95% unless indicated otherwise.

**Procedure B: Synthesis of Benzamidine-Containing Peptides (33a–b, 35, 42a–b, 45a–b).** Benzamidine-containing peptides were synthesized in analogy to the synthesis of amidines described previously. In short, peptide synthesis was performed according to procedure A. The peptide-substituted resin was swollen for 2 h in THF. Then 10 equiv of 1 M hydroxylamine hydrochloride solution was added and stirred at 60 °C for 16 h. The resin was washed with MeOH, THF, and DMF. Then 15 equiv mL of 1 M SrCl2/H2O was added under nitrogen atmosphere and stirred gently for 3 days at 80 °C. The substituted resin was washed with DMF, 5% DIPEA in MeOH, MeOH, DCM, and DEE. After cleavage and precipitation in cold diethyl ether, the peptides were purified by preparative RP-HPLC as described in procedure A. All peptides were freeze-dried and characterized by HR-ESI and 1H NMR for selected compounds (analytical data are provided in the Supporting Information). The purity was determined by analytical HPLC with method A. All evaluated peptides were obtained with a purity of at least 95% unless indicated otherwise.

**Procedure C: Synthesis of Bz-(3-guanidino)-Phg-Lys-Phg-NH2 (37).** The peptide synthesis of 36 (Bz-(3-nitro)-Lys(boc)-Phg-NH2) was performed according to procedure A with some modifications. 25 was coupled with HATU (3.5 equiv), HOAt (3.5 equiv), and disopropylethylamine (5.0 equiv) to the dipeptide sequence on solid support. After 1.5 h coupling time, the resin was washed with DMF, DCM, and again DCM to obtain 36. Then tinn(II) chloride dihydrate (60.0 equiv, 815 mg) and disopropylethylamine (20.0 equiv, 200 μL) in 2 mL of DMF was added to the resin and shaken overnight. The resin was washed with DMF, DCM, and DF. A mixture of bis-boc-pyrazole-1-carboxamide (8.0 equiv, 150 mg) and DMAP (3.0 equiv, 20 mg) in 2 mL of DMF was added and shaken overnight. The resin was washed with DMF, DCM, and DEE. The resin was dried overnight, and the peptide was cleaved using the standard procedure (92.5% TFA, 5.0% TIPS, and 2.5% water). The product was purified by preparative RP-HPLC as described in procedure A. The peptide was freeze-dried and characterized by HR-ESI and 1H NMR (analytical data are provided in the Supporting Information). The purity was determined by analytical HPLC with method A. Compound 37 was obtained with a purity of at least 95%.

**Synthesis of Bz-(4-acetamido)-Phe-Lys-Phg-NH2 (39).** Bz-(4-Dde(NH)Phe-(NHboc)lys-Phg-NH2 (38) was synthesized according to the Fmoc protocol described in procedure A. 37-substituted resin (120 mg; scale: 0.063 mmol) was swelled for 1 h in DCM. Then 2% hydrazine-DMF solution was used to cleave the ddi group (4 × 15 min). Ethylacetamidate hydrochloride59 (16 mg, 0.13 mmol) and disopropylethylamine (43 μL, 0.25 mmol) were dissolved in 1 mL of DMF and filled into the syringe. The reaction mixture was stirred at room temperature overnight. The substituted resin was washed with DMF, DCM, and DEE. The resin was dried overnight, and the peptide was cleaved by the standard procedure (92.5% TFA, 5.0% TIPS, and 2.5% water). The product was purified by preparative RP-HPLC as described in procedure A. The peptide was freeze-dried and characterized by HR-ESI. The purity was determined by analytical HPLC with method A (analytical data are provided in the Supporting Information).

**Synthesis of Bz-(4-acetamido)-Phe-Lys-Phg-NH2 (40).** 38 was synthesized according to the Fmoc protocol described in procedure A. The 38-substituted resin (117 mg; scale: 0.063 mmol) was swelled for 1 h in DCM. Then 2% hydrazine–DMF solution was used to cleave the ddiiv group (4 × 15 min). Acetic anhydride (24 μL, 0.25 mmol) and disopropylethylamine (43 μL, 0.25 mmol) were dissolved in 0.5 mL of DMF and filled into the syringe. The reaction mixture was stirred for 1 h. The substituted resin was washed with DMF, DCM, and DEE. The resin was dried overnight, and the peptide was cleaved by the standard procedure (92.5% TFA, 5.0% TIPS, and 2.5% water). The product was purified by preparative RP-HPLC as described in procedure A. The peptide was freeze-dried and characterized by HR-ESI and 1H NMR. The purity was determined by analytical HPLC with method A (analytical data are provided in the Supporting Information).

**Amino Acid Synthesis. Synthesis of (3-Nitro)-L-phenylglycine (22).** To a solution of L-phenylglycine (25 g, 165 mmol) in concentrated sulfuric acid (80 mL) was added a mixture of nitric acid (65%, 30 mL) and concentrated sulfuric acid (2.5 mL) dropwise at 0 °C. The resulting mixture was kept at 0 °C overnight and then poured on ice. Aqueous ammonia solution (25%) was added until the pH reached a value of 5. The resulting precipitate was collected and washed with water and ethyl alcohol. After recrystallization from ethanol (500 mL), crude 22 was obtained as a colorless solid (yield: 15.1 g, 47%). 1H NMR (300 MHz, DMSO-d6): δ = 4.25 (d, J = 6.2 Hz).
H2, 1H), 7.40–7.88 (m, 2H), 8.08–8.40 (m, 2H) ppm. HRMS (ESI): m/z [M + H]+ calcd for C4H9CN2O4, 211.0713; found, 211.0718.

**Procedure D: Synthesis of Methyl Ester Amino Acids.** 21 (4-Cyano)-l-phenylalanine Methyl Ester Hydrochloride (8a). The amino acid (0.50 g, 2.63 mmol) was dissolved in methanol (10 mL) and cooled on ice (0 °C). The THF solution (0.53 mL, 13.14 mmol) was added dropwise. The reaction mixture was warmed to room temperature and stirred overnight. The mixture was then concentrated in vacuo. No further purification was necessary (yield: 626 mg, quant.). *H NMR (300 MHz, CDCl3): δ = 3.84 (s, 3H), 5.48 (s, 1H), 7.78 (t, J = 8.2 Hz, 1H), 7.92 (m, 1H), 8.37 (dd, J = 2.2, 1.3 Hz, 1H), 8.44 (t, J = 2.2 Hz, 1H) ppm. HRMS (ESI): m/z [M + H]+ calcd, 205.0975; found, 205.0977.

- (3-Nitro)-l-phenylglycine Methyl Ester Hydrochloride (23). Thionyl chloride (6 mL, 83 mmol) was added dropwise to methanol (60 mL) at 0 °C. The mixture was allowed to warm to room temperature before 22 (11.7 mg, 59 mmol) was added. The solution was refluxed for 1 h, and the solvent was totally evaporated. The resulting residue was recrystallized from methanol/diethyl ether to obtain the hydrochloride of 22 as a colorless solid (yield: 82% g, 56%). *H NMR (300 MHz, CDCl3): δ = 3.84 (s, 3H), 5.48 (s, 1H), 7.78 (t, J = 8.2 Hz, 1H), 7.92 (m, 1H), 8.37 (dd, J = 2.2, 1.3 Hz, 1H), 8.44 (t, J = 2.2 Hz, 1H) ppm. HRMS (ESI): m/z [M + H]+ calcd, 205.0975; found, 205.0977.

1H NMR (300 MHz, CDCl3): δ = 7.75 (d, J = 8.37 Hz, 2H), 7.48 (d, J = 8.37 Hz, 2H), 4.40–4.46 (m, 1H), 3.81 (s, 3H), 5.07–5.17 (m, 1H), 3.38 (d, J = 6.17 Hz, 1H), 3.25 (d, J = 7.19 Hz, 1H) ppm. HRMS (ESI): m/z [M + H]+ calcd, 205.0975; found, 205.0977.

**Procedure E: Synthesis of N-benzoyl Methyl Ester Amino Acids.** N-Benzoyl-(4-aminomethyl)-L-phenylalanine Methyl Ester Tri-fluoroacetate (10b). A mixture of 18 (0.83 g, 2.53 mmol) and palladium on carbon (10%, 0.15 mmol) in acetic acid (1N, 5 mL) was stirred at room temperature under hydrogen pressure. No further purification was necessary. White-yellowish solid (yield: 421 mg, 50%). *H NMR (300 MHz, D2O): δ = 7.71–7.78 (m, 2H), 7.51–7.60 (m, 2H), 7.48 (s, 1H), 7.39–7.46 (m, 4 H), 6.69 (d, J = 7.05 Hz, 1H), 5.05–5.14 (m, 1H), 3.80 (s, 3H), 3.20–3.42 (m, 2H) ppm. 19F NMR (282 MHz, D2O): δ = −75.65. HRMS (ESI): m/z [M + Na]+ calc, 335.1366; found, 335.1370.

**Procedure F: Synthesis of N-Benzoyl-(4-amino)-L-phenylalanine Methyl Ester Hydrochloride (19).** A mixture of 18 (1.00 g, 3.15 mmol) and palladium on carbon (10%, 0.15 mmol) in acetic acid (1N, 5 mL) was stirred at room temperature under hydrogen pressure. No further purification was necessary. Yellowish oil (yield: 565 mg, 196%). 1H NMR (300 MHz, CDCl3): δ = 7.41–7.52 (m, 2H), 7.42–7.54 (m, 2H), 7.56 (m, 5H), 7.74 (m, 2H) ppm. HRMS (ESI): m/z [M + Na]+ calcd, 335.1366; found, 335.1370.

N-Benzoyl-(3-amino)-l-phenylglycine Methyl Ester Hydrochloride (3). Synthesized according to procedure F. Pale-red solid (yield: 130 mg, 41%). *H NMR (300 MHz, CDCl3): δ = 7.77–7.89 (m, 2H), 7.37–7.56 (m, 4 H), 7.22–7.30 (m, 3H), 4.88 (dd, J = 8.88, 5.36 Hz, 1H), 4.40 (s, 2H), 3.09–3.31 (m, 2H), 1.95 (s, 3H) ppm. HRMS (ESI): m/z [M + H]+ calc, 313.1547; found, 313.1550.

N-Benzoyl-(4-amino)-l-phenylalanine Methyl Ester Tri-fluoroacetate (11b). A mixture of 19 (0.83 g, 2.53 mmol) and palladium on carbon (10%, 0.15 mmol) in acetic acid (1N, 5 mL) was stirred at room temperature under hydrogen pressure. No further purification was necessary. White-yellowish solid (yield: 750 mg, 89%). *H NMR (300 MHz, CDCl3): δ = 7.69–7.77 (m, 2H), 7.48–7.57 (m, 1H), 7.39–7.48 (m, 2H), 7.34 (d, J = 8.37 Hz, 2H), 7.14 (d, J = 8.37 Hz, 2H), 4.90 (s, 1H) ppm. HRMS: m/z [M + Na]+ calc, 399.1390; found, 399.1393.

N-Benzoyl-(3-amino)-l-phenylglycine Methyl Ester Hydrochloride (26). Synthesized according to procedure F with some modifications. A mixture of 14 (1.7 g, 5.4 mmol) and palladium on carbon (10%, 0.40 mm, 0.38 mmol) in methanol (50 mL) and aqueous hydrochloric acid (1N, 5 mL) was stirred at room temperature under hydrogen atmosphere (1 bar) overnight. The mixture was filtered using Celite, the solvent was evaporated, and the crude hydrochloride 26 was obtained as a pale-red solid (yield: 1.71 g, 85%). *H NMR (300 MHz, CDCl3): δ = 3.78 (s, 3H), 5.84 (s, 1H), 7.39 (dt, J = 7.3, 2.0 Hz, 1H), 7.44–7.52 (m, 2H), 7.54–7.62 (m, 4H), 7.87 (2H) ppm. HRMS (ESI): m/z [M + Na]+ calc, 335.1366; found, 335.1370.

1H NMR (300 MHz, CDCl3): δ = 7.75 (d, J = 8.37 Hz, 2H), 7.48 (d, J = 8.37 Hz, 2H), 4.40–4.46 (m, 1H), 3.81 (s, 3H), 5.07–5.17 (m, 1H), 3.38 (d, J = 6.17 Hz, 1H), 3.25 (d, J = 7.19 Hz, 1H).
N-Benzoyl-(4-OTF)-L-phenylglycine Methyl Ester (15). Phenyl trifluoride (324 mg, 1.47 mmol) was added to a solution of 14 (490 mg, 1.33 mmol) and disopropylamine (580 μL, 3.33 mmol) in 50 mL of dry DCM on ice at 0 °C under N₂ atmosphere. The reaction mixture was warmed to room temperature and stirred overnight. The resulting product was dried under reduced pressure and purified by column chromatography on silica gel to give a colorless oil which crystallized on standing at room temperature. Colorless oil (yield: 544 mg, quant). 1H NMR (300 MHz, CDCl₃): δ = 7.50–7.88 (m, 2 H), 7.51–7.60 (m, 3 H), 7.43–7.50 (m, 2 H), 7.28–7.33 (m, 2 H), 5.82 (d, J = 6.61 Hz, 1 H), 3.81 (s, 3 H) ppm. 13C NMR (75 MHz, CDCl₃): δ = −72.87 (CF₃) ppm. HRMS (ESI): [M + Na]⁺ calcd, 317.0897; found, 317.0887.

N-Benzoyl-(3-cyano)-L-phenylglycine Methyl Ester (16).25,26 Bis(triphenylphosphine)nickel bromide (94 mg, 0.13 mmol), triphenylphosphine (99 mg, 0.38 mmol), zinc powder (91 mg, 1.39 mmol), and diisopropylethylamine (580 μL, 3.33 mmol) were dissolved in dry acetonitrile (4 mL) and added dropwise. A mixture of crude alanine Methyl Ester (490 mg, 1.33 mmol) and sodium hydrogen carbonate (0.15 g, 1.76 mmol) were added. The reaction mixture was then evaporated under reduced pressure. The resulting product was dissolved in dry acetonitrile (4 mL) and added dropwise. The reaction mixture was stirred suspension of magnesium sulfate, and evaporated under reduced pressure. The resulting product was dissolved in dry acetonitrile (4 mL) and added dropwise. The reaction mixture was then evaporated under reduced pressure. The resulting product was dissolved in dry acetonitrile (4 mL) and added dropwise. The reaction mixture was then evaporated under reduced pressure. 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The resulting product was dissolved in dry acetonitrile (4 mL) and added dropwise. The reaction mixture was then evaporated under reduced pressure. The resulting product was dissolved in dry acetonitrile (4 mL) and added dropwise. The reaction mixture was then evaporated under reduced pressure.
(yield: 426 mg, 36%). 1H NMR (300 MHz, CDCl3): δ = 7.72 (d, J = 7.93 Hz, 2 H), 7.39–7.56 (m, 3 H), 7.30 (s, 1 H), 7.07 (s, 2 H), 6.70 (d, J = 7.05 Hz, 1 H), 5.06–5.16 (m, 1 H), 3.26–3.30 (m, 2 H), 2.91–3.03 (m, 2H), 2.47 (s, 3 H), 1.74–1.90 (m, 1 H), 1.08 (s, 4 H), 0.83–1.00 (m, 5 H), 0.74 (d, J = 6.61 Hz, 4 H) ppm. HRMS (ESI): [M + H]+ calcd, 489.2395; found, 489.2422.

N-Benzyl-(3-NO2)-c-phenylglycine (25). To a solution of 24 (200 mg, 0.64 mmol) in THF (20 mL) at 0 °C was added a cold solution of lithium hydroxide (100 mg, 4 mmol) in water (20 mL), and the mixture was stirred at 0 °C for 1 h. Aqueous hydrochloric acid (1N, 10 mL) and water (10 mL) were added, and the remaining THF was evaporated. The resulting precipitate was collected, washed with water, and dried to obtain 25 as a pale-yellow solid (yield: 140 mg, 73%). 1H NMR (300 MHz, CD3OD): δ = 5.88 (s, 1H), 7.48 (m, 2H), 7.57 (m, 1H), 7.65 (t, J = 8.0 Hz, 1H), 7.90 (m, 3H), 8.23 (ddd, J = 8.2, 2.3, 1.1 Hz, 1H), 8.42 (t, J = 2.2 Hz, 1H) ppm. HRMS (ESI): m/z [M + H]+ calcd for C26H31N4O7, 511.2198; found, 511.2201.

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■ ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free on the ACS Publications website at DOI: 10.1021/acs.jmedchem.5b00612.

Inhibitory activity of compound 47 against DENV and WNV proteases, thrombin and trypsin, and detailed results for the aprotinin competition assay; results of the permeability testing (PAMPA); detailed results for the synthesis of peptide hybrids, HR-MS and HPLC data for all synthesized peptides used in biochemical and biological evaluations, NMR data for selected peptides, and analytical data for synthetic intermediates (PDF)

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■ ABBREVIATIONS USED

Abz, aminobenzoic acid; Ddiv, 1-(4,4-dimethyl-2,6-dioxycyclo-hex-1-ylidene)-3-methylbutyl; DENV, dengue virus; DIPEA, diisopropylethylamine; HATU, O-(7-azabenzoatriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate; HOAt, 1-hydroxy-7-azabenzoazole; MOI, multiplicity of infection; PAMPA, parallel artificial permeability assay; TFHF, fluoro-N,N,N′,N′-tetramethylformamidinium hexafluorophosphate; THR, thrombin; TRY, trypsin; WNV, West Nile virus

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